Identifying qualitative effects of different grazing types on below-ground communities and function in a long-term field experiment

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Summary

Herbivory is an important modulator of plant biodiversity and productivity in grasslands, but our understanding of herbivore-induced changes on below-ground processes and communities is limited. Using a long-term (17 years) experimental site, we evaluated impacts of rabbit and invertebrate grazers on some soil functions involved in carbon cycling, microbial diversity, structure and functional composition. Both rabbit and invertebrate grazing impacted soil functions and microbial community structure. All functional community measures (functions, biogeochemical cycling genes, network association between different taxa) were more strongly affected by invertebrate grazers than rabbits. Furthermore, our results suggest that exclusion of invertebrate grazers decreases both microbial biomass and abundance of genes associated with key biogeochemical cycles, and could thus have long-term consequences for ecosystem functions. The mechanism behind these impacts are likely to be driven by both direct effects of grazing altering the pattern of nutrient inputs and by indirect effects through changes in plant species composition. However, we could not entirely discount that the pesticide used to exclude invertebrates may have affected some microbial community measures. Nevertheless, our work illustrates that human activity that affects grazing intensity may affect ecosystem functioning and sustainability, as regulated by multi-trophic interactions between above- and below-ground communities.

Introduction

Grasslands comprise approximately 40% of the earth's land area (Briske and Richards, 1995), and are important agri-environments because they carry out key ecosystem services, including forage production and carbon storage. They also contribute to biodiversity, nutrient cycling, and climate and pollution regulation (Tilman *et al*., 1996; Lal *et al*., 2007). Grasslands typically support a large, diverse and active microbial community as a result of high plant diversity, C inputs and turnover. Like other ecosystems, in grasslands, above- and below-ground communities are intimately connected through numerous interactions that are important factors governing ecosystem functioning. It is important to gain a more complete understanding of the interactions between above- and below-ground communities to better establish the consequences of human activity for ecosystem functioning and sustainability, and to develop appropriate management and conservation polices (Kremen, 2005).

Terrestrial microbial communities play a critical role in such above- and below-ground interactions because of their influence in maintaining ecosystem services. This is driven largely by their ability to carry out multiple functions, including biogeochemical cycling of carbon (C) and nitrogen (N) (Balser and Firestone, 2005), disease suppression (Mendes *et al*., 2011), and pollutant removal (Kuiper *et al*., 2004). It has been well established that both the composition and functional capabilities of soil microbial communities affect plants (Bardgett and Wardle, 2010), and that interactions between above- and below-ground communities influence ecosystem functioning through feedback mechanisms (Bardgett, 2005). Herbivore grazing is a key regulator of above-ground productivity and diversity in grassland ecosystems (McNaughton *et al*., 1989; Crawley, 1990; Collins *et al*., 1998), and thus could also indirectly impact below-ground communities in the short-term by influencing resource allocation via altered litter inputs and stimulating below-ground carbon allocation (Bardgett *et al*., 2001; Hamilton and Frank, 2001), or in the long-term by altering the organic matter turnover (Bardgett and Wardle, 2010). Further, longer term plant-soil feedback may be realized as a result of enhanced litter quality through either herbivore-mediated selection of plant species with lower leaf C : N (Rossignol *et al*., 2011) or increased N-uptake by plants with subsequent increase in leaf litter quality (Briske and Richards, 1995; Chapman *et al*., 2003), which ultimately could increase soil N availability (Hamilton and Frank, 2001). Because C and/or N availability generally limit soil microbial communities, such herbivore-mediated changes are likely to influence microbial biomass and activity. Indeed, some studies assessing the impact of large vertebrate grazers have demonstrated increased soil C and N availability, increased microbial biomass, and changes in microbial activity and diversity (Zhou *et al*., 2010a; Liu *et al*., 2011; Shan *et al*., 2011). Such impacts are believed to be a result of grazing-mediated changes in root exudation patterns and root turnover (Holland *et al*., 1996; Bardgett and Wardle, 2003; Hamilton *et al*., 2008; Olsen *et al*., 2011). It has also been suggested that different grazing intensities may alter fungal to

bacterial ratios in soils (Bardgett *et al*., 1996; 1997; 1998), altering energy flows, rates of nutrient cycling and consequently potential for C-storage within grasslands ecosystems.

To date, most studies assessing the impacts of herbivory on below-ground soil communities and processes in grassland systems have been concerned with large vertebrate grazers, including sheep and ungulates (e.g. Bardgett *et al*., 1997; Hamilton and Frank, 2001; Liu *et al*., 2012; Stark *et al*., 2012). However, natural grasslands are subject to continuous or intermittent grazing from smaller vertebrate herbivores, including rabbits, as well as from invertebrate herbivores. It has been estimated that vertebrate and invertebrate grazers could consume up to 50% of above-ground biomass in grassland systems (Dellting, 1988), which would significantly influence nutrient flows and microbial activity and turnover. Rabbit grazing is important for the maintenance of grasslands in southern England (Crawley, 1990), and there is compelling evidence to support the view that invertebrates are also important drivers of plant community composition in grasslands (Allan and Crawley, 2011). Despite this, few experiments have assessed the impacts of different types of herbivore grazers on below-ground communities and processes (Risch *et al*., 2013). Because herbivory encompasses both vertebrate (large and small) and invertebrate grazers that may affect numerous ecosystem properties and functions differently, an improved understanding of the influence of different herbivore grazers on soil processes and microbial communities is important if we are to effectively identify drivers of ecosystem functioning in order to develop policies concerning conservation, restoration and sustainability.

Currently, there is a lack of long-term studies that consider multiple grazers in natural field conditions. Such information is critical to advance our scientific understanding of multi-trophic interaction and ecosystem functions. The aim of this study was to examine the long-term (17-year) impact of rabbit and invertebrate grazers, and their interaction, on key soil functions, community structure and biodiversity. It was hypothesized that long-term grazing would increase below-ground carbon allocation and alter nutrient (nitrogen and phosphorus) availability, resulting in increased microbial biomass and activity, and changes in the community composition, which in turn would impact ecosystem functioning. Additionally, it was hypothesized that the effects would be more pronounced under higher grazing pressure (rabbit) compared with moderate (insect and mollusc) grazing. To test these hypotheses, we used a perennial grassland exposed to 17 years of vertebrate (rabbit) and/or invertebrate (insects, molluscs or insect + mollusc) herbivore exclusion, which had resulted in significant changes in above-ground plant species richness, composition and productivity (Allan and Crawley, 2011). The fully factorial experimental design (Allan and Crawley, 2011) allowed for progressive elimination of grazing pressure by selectively excluding mollusc, insect, mollusc + insect, or rabbit grazers, allowing a

novel approach to identify the impacts of different grazer groups on soil microbial community and functions.

Results and discussion

From herein, the following are used to define treatments: ‘F’ is fenced, to exclude rabbit grazers; ‘R’ is rabbit grazers present; ‘+ IM’ is insect and mollusc grazers present; ‘+ I’ in insect grazers only (i.e. no molluscs); ‘+ M’ is mollusc grazers only (i.e. no insects); and ‘− IM’ is no insect or mollusc grazers.

Key ecosystem functions responses

Functional activities (basal respiration and C-substrate utilization) were tested using MicroResp™ (Campbell *et al*., 2003). Utilization of a number of C-substrates was significantly impacted in R treatments, namely a decrease in galactose, glucose, malic acid and α-ketoglutaric acid-induced respiration rates (Supporting Information Table S1). Rabbits reduce above-ground plant production by > 70% at this site (Olofsson *et al*., 2007), and a reduction in the utilization of carbon sources associated with structural plant carbohydrates in these soils may reflect reduced levels of above-ground biomass entering the decomposition pathway, as well as changes in plant species composition because less palatable plant species, with more recalcitrant litter, are likely to dominate by selective grazing on more palatable species in rabbit-grazed plots (Crawley, 1983; 1990; Bardgett and Wardle, 2003). Although annual removal of standing biomass occurred in F treatments, rabbit grazing is continual throughout the year, and is likely to have more of an impact on the quality and quantity of carbon (and other nutrient) returns to the system than an annual removal event. Invertebrate grazing (+ IM, + I, + M treatments) had a lesser effect of C-utilization, although the utilization of oxalic acid was lower in these soils compared with − IM treatments where invertebrate grazers had been excluded (Supporting Information Table S1). Oxalic acid is essential for the formation of calcium oxalate and is believed to play an important role in the protection against herbivory (Franceschi and Nakata, 2005). There was no overall impact of R, + I, + M or + IM treatments on total heterotrophic respiration (Supporting Information Table S1).

Microbial biomass and broad-scale community structure were assessed using the phospholipid fatty acid (PLFA) analysis (Frostegard *et al*., 1993). Although there was no effect of R treatment on either total biomass or the biomass of specific microbial groups (Table 1), there was a significantly lower biomass of all bacterial groups and the arbuscular mychorzhizal (AM) PLFA marker in − IM treatments compared with soils with invertebrate grazing (i.e. + I, + M, + IM treatments) ($P < 0.001$; Table 1). The most notable observation was that the progressive exclusion of invertebrate grazers leads to lower total PLFA, whereby total PLFA was 38%, 50% and 53% lower in + I, + M and − IM treatments, respectively, compared with + IM treatments. This significantly higher total PLFA biomass in + IM treatments was consistent with higher % C

and % N in these soils compared with − IM treatments where invertebrate grazers were suppressed (Supporting Information Table S2 and Fig. S1). This observation was independent of whether rabbits were present (R) or absent (F), and suggests that the presence of insects and molluscs increased C and N flow into soils, presumably through increased litter production and/or root exudation, as has been previously reported for grassland systems (Holland *et al*., 1996; Hamilton and Frank, 2001), and through the presence of insect excreta, stimulating microbial biomass and activity (Bardgett and Wardle, 2003). The trend of higher PLFA in + IM treatments was consistent across all bacterial groups. Generally, + M or − IM treatments had significantly lower PLFA across all microbial groups. The impact of + I treatment was less severe, suggesting that insect grazers had a greater positive impact than mollusc grazers. Notably, the PLFA associated with AM fungi was higher in invertebrate-grazed plots (+ I, + M, + IM treatments) than in − IM treatments (Table 1). This observation is likely to be driven by herbivore-mediated changes in the quantity and quality root exudates under herbivore grazing (Olofsson *et al*., 2007), thus increasing C resource for colonizing AM fungi. The fact that there was no impact of R treatment on PLFA biomass was unexpected considering rabbit droppings in R treatments would have likely resulted in more carbon and nutrients being returned to the decomposition pathway compared with − R plots, where standing vegetation was removed annually. The higher levels of variability seen between replicates across R treatments compared with F treatments is likely to reflect that patchy nature of vegetation and rabbit urination and droppings experienced in grazed soils, and these results are in keeping with the highly idiosyncratic nature of vertebrate herbivore effects on soil biota and soil processes previously reported in the literature (Bardgett *et al*., 2001; 2003; Wardle *et al*., 2004; Risch *et al*., 2013). Previous work at this site demonstrated that insect grazers had a significant positive effect on plant species richness, and that molluscs significantly reduced herb abundance (Allan and Crawley, 2011). Taken together, our data suggest that invertebrate grazers impact microbial biomass via an impact on plant diversity, while higher grazing intensity by rabbits did not.

Table 1. Mean phospholipid fatty acid (nmol g^{-1}) of different microbial groups in fenced (F) and rabbit-grazed (R) soils with either insect + mollusc grazing (+ IM), insect grazing (+ I), mollusc grazing (+ M) or no invertebrate (IV) grazing (– IM) Numbers in parenthesis are =/1 one standard error.

	Invertebrate (IV) treatment										
	+ IM		+ I		+ M		– IM		Rabbit grazers	Invertebrate grazers	
PLFA group	F	R	F	R	F	R	F	R	(R)	(IV)	R × IV
Total	156.2 (19)	209.3 (37)	109.2 (8.1)	117.1 (12)	92.3 (15)	89.6 (27)	88.6 (12)	82.0 (25)	NS	$P < 0.001$	NS
Bacterial	90.1 (11)	118.1 (22)	61.1 (4.6)	65.8 (6.8)	50.6 (8.9)	49.9 (15)	49.2 (7.1)	45.1 (13)	NS	$P < 0.001$	NS
Fungal	0.90 (0.7)	3.40 (1.4)	0.02 (0.00)	0.57 (0.4)	0.60 (0.6)	0.02 (0.00)	0.45 (0.5)	1.36 (1.9)	NS	NS	NS
F : B	0.0097 (0.01)	0.0256 (0.01)	0.0003 (0.00)	0.0119 (0.01)	0.0174 (0.02)	0.0006 (0.00)	0.0096 (0.01)	0.0323 (0.03)	NS	NS	NS
Actino	7.19 (1.0)	9.72 (1.5)	4.86 (0.4)	6.17 (0.7)	4.51 (0.8)	4.85 (1.1)	4.37 (0.7)	3.81 (1.0)	NS	$P < 0.001$	NS
G+	28.1 (5.1)	38.9 (8.5)	17.5 (1.5)	20.5 (2.3)	13.3 (2.9)	14.7 (4.6)	14.9 (3.0)	13.9 (3.8)	NS	$P < 0.001$	NS
G–	61.9 (6.6)	78.1 (13)	43.6 (3.5)	45.3 (5.1)	37.2 (6.3)	34.9 (10)	34.3 (4.3)	31.2 (9.6)	NS	$P < 0.001$	NS
G+ : G–	0.442 (0.04)	0.484 (0.03)	0.407 (0.03)	0.467 (0.05)	0.342 (0.05)	0.397 (0.90)	0.416 (0.04)	0.568 (0.2)	NS	NS	NS
AM	5.31 (0.9)	7.02 (1.3)	3.79 (0.4)	3.55 (0.5)	3.06 (0.6)	2.34 (0.9)	2.75 (0.4)	2.30 (0.7)	NS	$P < 0.001$	NS

Values are means across fenced and rabbit-grazed plots. F : B is fungal bacterial ratio; actino is *Actinobacteria*; G+ is Gram-positive bacteria; G– is Gram-negative bacteria; AM is *Arbuscular mycorrhizae*.

Biodiversity and community structure response

Biodiversity and community structure of the microbial communities were carried out using 454 sequencing and multiplex terminal restriction fragment length polymorphism (M-TRFLP) analyses (Singh *et al*., 2006; 2014). Analysis of variance (ANOVA) of M-TRFLP principal component (PC) scores demonstrated a significant effect of R treatment on bacterial community structure (Supporting Information Table S3). Strong, significant differences in bacterial community structure between R and F treatments were evident on the first PC ($P < 0.001$), which accounted for a large percentage of the variance (36.6%), whereby communities associated with R treatments separated to the left of those associated with F treatments (Fig. 1A). Invertebrate grazing (+ I, + M and + IM treatments) also impacted on bacterial community structure with differences on the second, third, fourth and fifth PC dimensions (Supporting Information Table S3), although there was clear separation of F + IM treatments from F − IM treatments on the second PC, and of R + IM treatments from R − IM treatments on the first and second PC dimensions (Fig. 1A). Further, within R treatments, there was a progressive shift in bacterial community structure on the second PC with increasing level of invertebrate grazing (Fig. 1A). The fungal community was largely unaffected by rabbit or invertebrate grazers alone (Supporting Information Table S3), although there was a weak, but significant, interaction between R + IM treatments (Supporting Information Table S3), separating the fungal community associated with R + IM treatments from that associated with F + IM treatments (Fig. 1B). These results indicate that at the broad scale, fungal community was generally less responsive to rabbit or invertebrate grazers. This observation is supported by the PLFA data. There was some evidence that higher C : N ratios in rabbit-grazed soils correlated with a shift in bacterial community structure (Supporting Information Fig. S2).

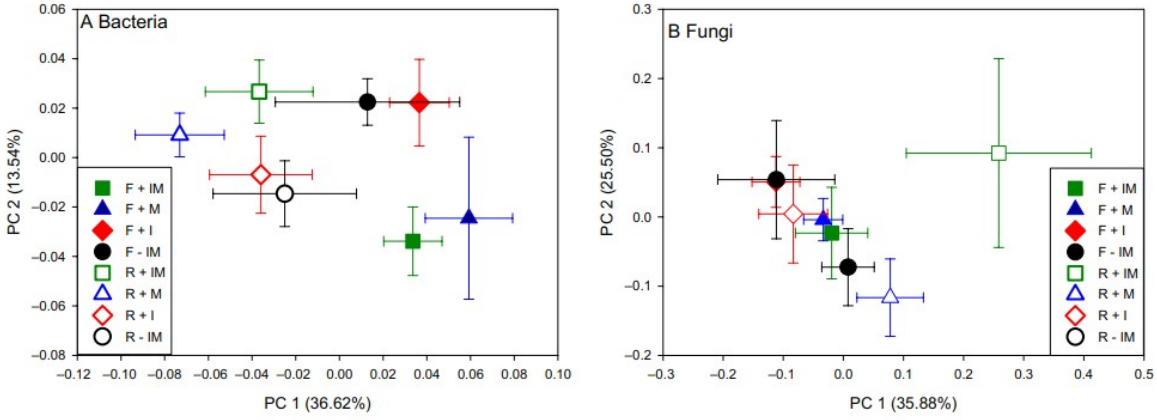

Fig. 1. Biplot of principal component (PC) scores of (A) bacterial and (B) fungal community structure in soils under different herbivory regimes as assessed by TRFLP analysis. Solid symbols are fenced plots; open symbols are rabbit-grazed plots; green symbols are insect + mollusc grazed (+ IM); blue symbols are mollusc-grazed (+ M); red symbols are insect-grazed (+ I); and black symbols have no invertebrate grazing (– IM). Numbers in parentheses are percentage variation explained by that principal component. Error bars are ± 1 SE ($n = 6$).

At a higher level of community resolution (454 pyrosequencing analysis), rarefaction analysis indicated that α-diversity remained unchanged under

both R and + IM treatments compared with F and − IM treatments respectively (Supporting Information Fig. S3). Community structure (β-diversity), on the other hand, was significantly impacted in R treatments compared with F treatments at the phylum, class and order level (Supporting Information Table S4; Fig. 2). The direction and magnitude of the response to R treatment differed between different microbial taxa (Fig. 2 and Supporting Information Fig. S4). Among the dominant taxa, *Acidobacteria* and *Firmicutes* were on average 11% and 47%, respectively, less abundant in R treatments compared with F treatments, while *Actinobacteria* and *Proteobacteria* were on average 23% and 10%, respectively, more abundant in R treatments compared with F treatments (Fig. 2B; Supporting Information Fig. S4A and B). Significant negative effects of R treatment on the relative abundance of some less dominant phyla (OP10 and OD1) were also observed (Supporting Information Fig. S4A). The positive response to R treatment within the *Proteobacteria* was mainly caused by an increase in the *β-Proteobacteria* (Supporting Information Fig. S4B). Within the *Acidobacteria*, negative effects were mainly driven by impacts on *Acidobacteria Gp1*, *2* and *3*. This is an interesting finding, and previous reports linked shift in relative dominance from *Acidobacteria* to *Proteobacteria* in ecosystem where nutrient availability had increased (Fierer and Jackson, 2006; Singh *et al*., 2010). *Proteobacteria* have been reported to belong to copiotrophs and grow comparatively faster where energy availability is abundant, while *Acidobacteria* are mainly considered oligotrophs (Fierer and Jackson, 2006; Singh *et al*., 2010), and the observed increase in the ratio between *Proteobacteria* and *Acidobacteria* ($P < 0.05$, data not shown) in R treatments fits the hypothesis that rabbit grazing causes increased C-allocation below-ground through increased root exudation. Together, these data support a model that nutrient turnover in R treatments occurs via a rapid decomposition pathway (via rabbit excreta) compared with a slower decomposition pathway of litter return in F treatments where rabbit grazing was excluded. Previous work on comparable rabbit enclosures at Silwood Park supports this model, whereby N-mineralization rates were significantly higher in grazed soils compared with soils where rabbits had been excluded for 14 years (Olofsson *et al*., 2007). Our findings suggest that more resource exchange takes place between above- and below-ground communities under the intensive grazing treatment. Indeed, the observation of increased soil nutrient availability and cycling under vertebrate grazing has been reported before for large herbivore grazers (Chaneton *et al*., 1996; McNaughton *et al*., 1997). However, in our study, the difference in soil pH and soil C : N ratio was comparatively small compared with previously published studies, and therefore it is likely that other variables such as plant species composition may also have a stronger influence on the microbial communities in these soils.

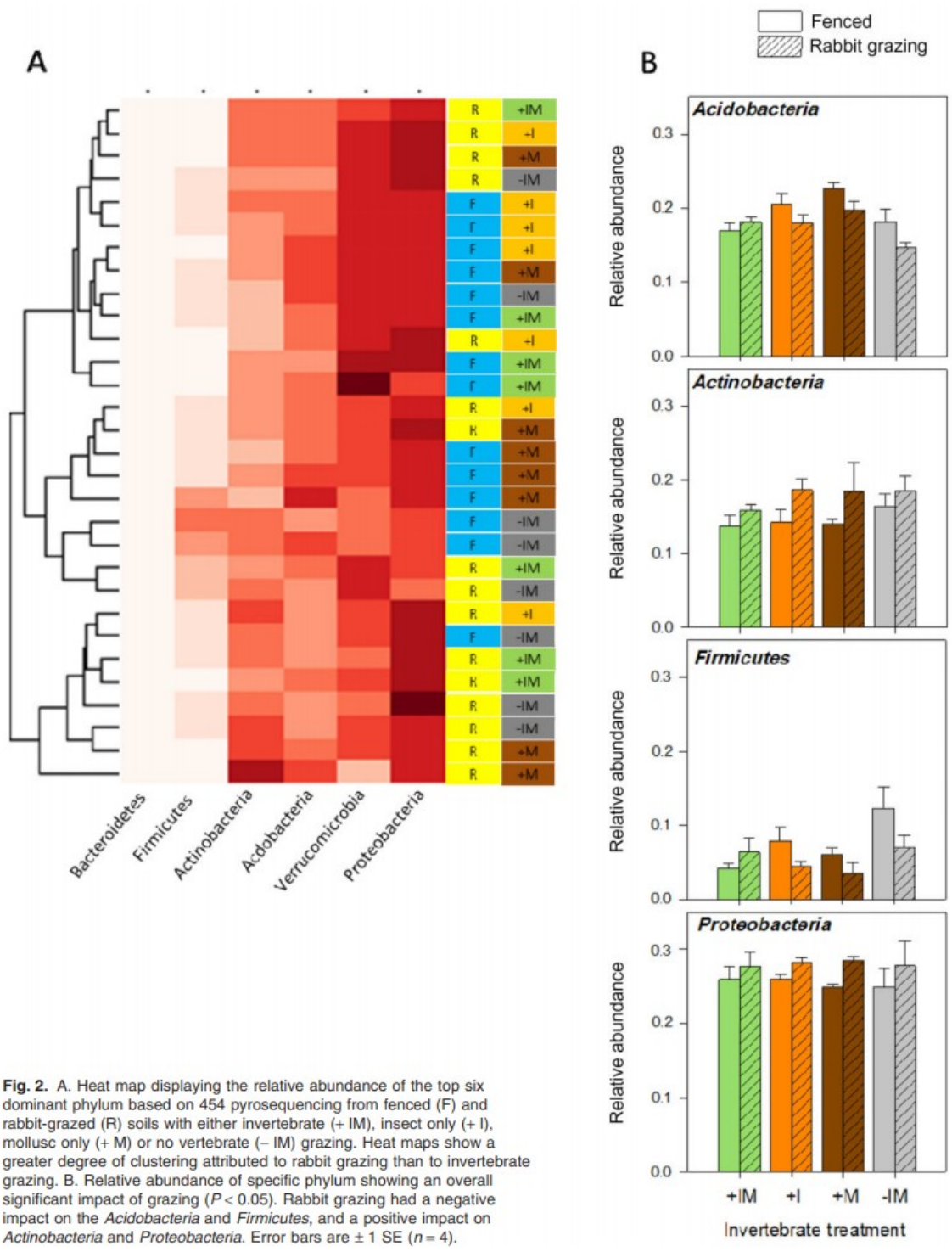

Fig. 2. A. Heat map displaying the relative abundance of the top six dominant phylum based on 454 pyrosequencing from fenced (F) and rabbit-grazed (R) soils with either invertebrate (+ IM), insect only (+ I), mollusc only (+ M) or no vertebrate (– IM) grazing. Heat maps show a greater degree of clustering attributed to rabbit grazing than to invertebrate grazing. B. Relative abundance of specific phylum showing an overall significant impact of grazing ($P < 0.05$). Rabbit grazing had a negative impact on the *Acidobacteria* and *Firmicutes*, and a positive impact on *Actinobacteria* and *Proteobacteria*. Error bars are ± 1 SE ($n = 4$).

Functional community responses

Functional communities were analysed using GeoChip functional gene array (He *et al.*, 2010). Full factorial comparisons were made among R, F, + IM and − IM treatments only. Because of the importance of C-, N-, P- and S-cycling in maintaining ecosystem health, analysis of genes associated with these

biogeochemical cycles could yield important insights into the long-term sustainability of different management practices. Together, this data provide information relating to the functional potential of a microbial community under different modes of herbivory. The relative abundance of a number of genes associated with biogeochemical cycles (C, N, P and S) was significantly higher in R treatments compared with F treatments (Supporting Information Table S5). Analysis of PC scores across gene categories revealed differential impact of R and of + IM treatments on functional gene compositions (Supporting Information Table S6 and Fig. S5), suggesting shifts in functional community structure, as supported by the TRFLP/pyrosequencing data. Considering the relative abundance of genes at the subcategory level within each category, ANOVA revealed significant effects of both R and of + IM treatments on the genes associated with specific processes (Supporting Information Table S7). For C-cycling, genes associated with methane oxidation were significantly lower in R treatments compared with F treatments (Fig. 3A). This was driven by a 25% reduction in the relative abundance of *pmoA* genes, and corresponded with a 20% increase in *mcrA* genes associated with methane production, alongside a 60% increase in the FTHFS genes associated with acetogenesis (Fig. 3A), a major substrate for methane production. This corresponded with a 15% increase in soil moisture levels in R treatments (Supporting Information Table S2), presumably providing more favourable conditions for anaerobic methane production. This finding is consistent with reports that higher soil moisture promotes methane emission by increasing methane production and decreasing methane oxidation (Singh *et al*., 2007; Nazaries *et al*., 2013). Acetogens are among the key precursors for methanogenesis pathways (Nazaries *et al*., 2013), and these results suggest that a number of variables in the methane cycle are impacted by rabbit grazers. The relative abundance of fungal *ara* genes involved in hemicellulose degradation was 20% lower in R treatments compared with F treatments, while bacterial mannanase genes also associated with hemicellulouse degradation were 30% higher in R treatments.

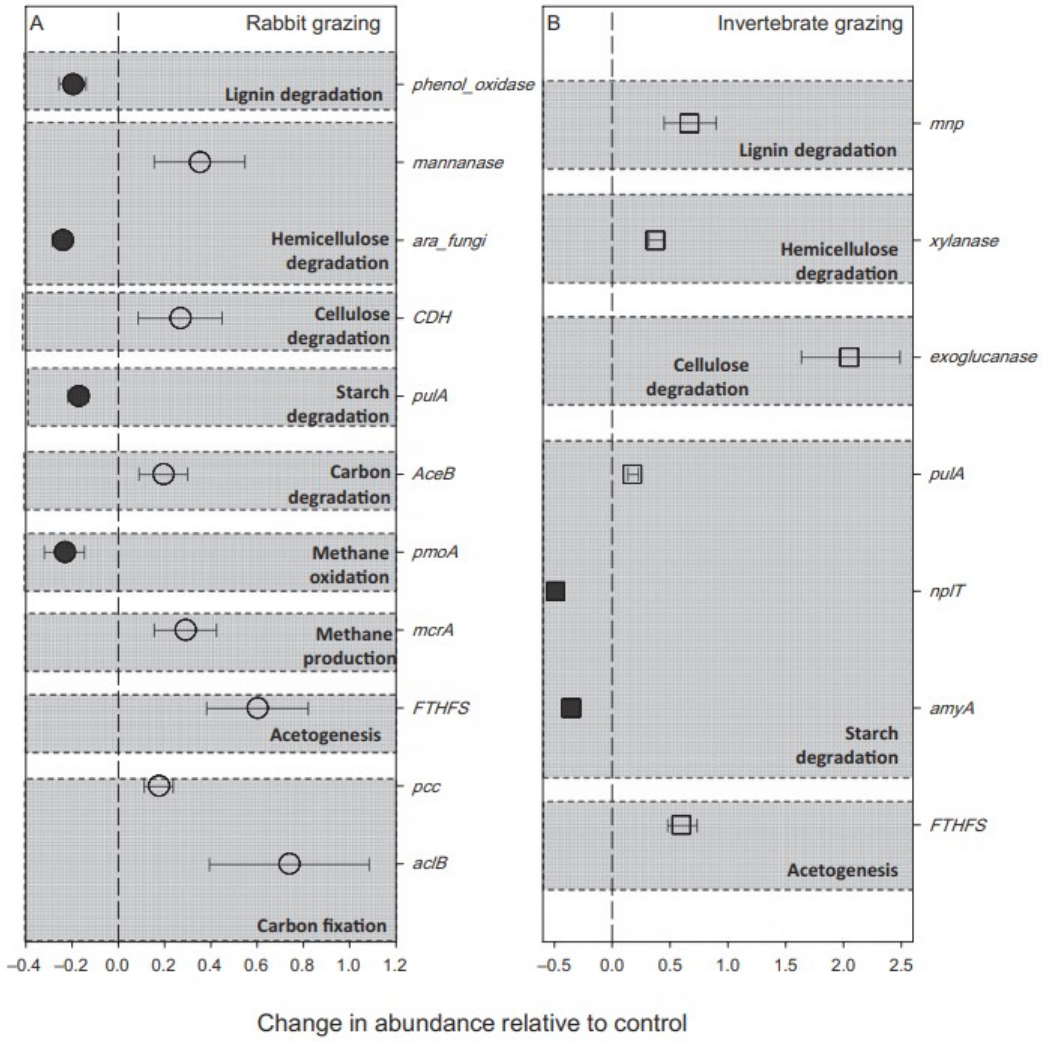

Fig. 3. Significant ($P < 0.05$) directional change (relative to ungrazed control) in relative abundance of genes associated with C-cycling in (A) rabbit-grazed soils and (B) invertebrate-grazed soils. Open symbols represent a positive effect of grazing, and closed symbols indicate a negative effect of grazing. Error bars are ± 1 SE ($n = 8$).

Genes associated with acetogenesis were significantly higher in + IM treatments compared with − IM treatments (Fig. 3A; Supporting Information Table S7), suggesting that invertebrate grazers promote the activity/presence of acetogens. The presence of invertebrate grazers (+ IM treatments) also impacted significantly at the gene category level (Supporting Information Tables S5 and S7). Genes associated with acetogenesis and cellulose degradation were higher in + IM treatments compared with − IM treatments (Fig. 3B). Cellulose is a major cell wall constituent, and it may be postulated that in the + IM treatments greater turnover of plant material via increased leaf damage and death could promote the abundance of genes associated with starch degradation. There was evidence that under R treatments, the relative abundance of some genes associated with starch degradation were lower than in F treatments. This differential response to + R and + IM treatments may be a reflective of

the different grazing behaviours. Rabbit grazing removes significant plant biomass from the system (Olofsson *et al.*, 2007), whereas invertebrate grazing will lead to increased litter input through plant damage and death. Conversely, genes associated with lignin and hemicellulose degradation were higher in + IM treatments compared with − IM treatments (Fig. 3B). A shift in plant species composition was observed under + IM treatments, whereby the proportion of herbs and legumes declined compared with − IM treatments (Allan and Crawley, 2011). As herbs and legumes generally have higher lignin contents than grasses (Khalsa *et al.*, 2012), mechanisms other than changes in plant species composition are likely to be driving the higher abundance of these genes in + IM treatments.

The + IM treatments had a bigger effect on the number of genes associated with N-cycling than + R treatments did (Supporting Information Table S5), but there was a strong influence of both + R treatment and + IM treatment on the functional composition of these genes (Supporting Information Table S6; Supporting Information Fig. S6). Although there were several genes associated with N-cycling affected by both + R and + IM treatments (Supporting Information Fig. S6), there was no apparent overall impact on any group of genes associated with a particular process within the N-cycle. For P-cycling, there was a strong positive effect of R treatment on the relative abundance of P-utilization genes (Supporting Information Fig. S7), suggesting increased P-mineralization under rabbit grazing. This was only evident, however, when invertebrates were also present (R + IM). The abundance of P-utilization genes was also higher in + IM treatments (Supporting Information Fig. S7). As discussed above, nutrient turnover in rabbit-grazed systems is likely to be more rapid compared with F treatments, due mainly to increased labile nutrient inputs via animal excreta. Higher nutrient turnover will, in turn, increase P demand and consequently increase P-mineralization rates as has been previously reported for Pampa grasslands grazed by large herbivores (Chaneton *et al.*, 1996). Genes associated with S-cycling also showed an increase in + IM treatments compared with − IM treatments (Supporting Information Fig. S8). The direction and magnitude of these effects varied between different genes (Supporting Information Fig. S8). Overall, GeoChip data closely aligned with PLFA data and molecular M-TRFLP and 454 data, where both rabbit and less intensive invertebrate grazing had significant impacts on community structure. In fact, there were very strong correlations between GeoChip and 454 PC scores across phylum, class and genus levels (Supporting Information Fig. S9), providing strong support that grazing impacts microbial community composition.

While ANOVA of GeoChip functional gene data was used to determine treatment effects on specific genes, and multivariate analysis was used to determine effects of grazing treatment on overall functional composition, we also employed a network analysis approach, based on random matrix theory (RMT; Deng *et al.*, 2012) to determine whether grazing treatment had a significant impact on the complex interactions among microbial functional

groups within a community. Significant differences (as determined by pair-wise comparisons, using standard deviations acquired from random networks) in the architecture of the microbial ecological networks were apparent (Table 2; Fig. 4). The network size and degree of connectivity were greater in both R treatments and + IM treatments than in F and − IM treatments (Table 2), and the structure and composition of these networks differed significantly, as measured by average connectivity, average clustering coefficient, average path lengths and modularity. A higher number of nodes, connectivity and number of modules in microbial communities from R and + IM, compared with F and – IM, respectively, indicates that both rabbit and invertebrate grazers invoke a higher degree of interaction between different organisms with a community. This observation is consistent with PLFA data where significantly lower biomass was observed in − IM, − I and − M treatments compared with + IM treatments, suggesting a consistent effect of invertebrate grazers on all measured microbial attributes, including biomass, structure and functional community composition.

Table 2. Architectural properties of microbial ecological networks across different vertebrate and invertebrate grazing treatments.

	Empirical networks								Random networks		
Community	No. of original genes	Similarity threshold	Network size (*n*)	*R* of scale free (significance)	Average connectivity (*avgK*)	Average path length (GD)	Average clustering coefficient (*avgCC*)	Modularity (no. of modules)	Average path length (GD)	Average clustering coefficient (*avgCC*)	Modularity (M)
F	1414	0.96	814	0.936	3.501	3.027^{a}	0.121^{a}	0.712^{a} (106)	3.878 ± 0.130	0.012 ± 0.002	0.559 ± 0.004
+ R	1683	0.96	977	0.865	4.925	3.887^{b}	0.198^{b}	0.668^{b} (115)	3.762 ± 0.079	0.013 ± 0.002	0.436 ± 0.003
– IM	1275	0.96	564	0.926	2.415	4.137^{x}	0.116^{x}	0.892^{x} (94)	3.937 ± 0.242	0.006 ± 0.003	0.731 ± 0.006
+ IM	1961	0.96	1144	0.939	3.367	3.686^{y}	0.113^{y}	0.748^{y} (111)	4.073 ± 0.100	0.011 ± 0.002	0.582 ± 0.003

F is fenced, + R is rabbit grazing, – IM is no invertebrate grazing and + IM is vertebrate grazing. For each network, $n = 8$, so that + R and F include samples from both + IM and – IM treatments, and similarly – IM and + IM include samples from both + R and F treatments. Pair-wise comparisons were made between F and + R, and between – IM and + IM, for selected empirical network properties. For these pair-wise comparisons, different letters indicated significant differences ($P > 0.001$).

There is a possibility that the impact we have reported for exclusion of invertebrate grazers on below-ground communities and functions is partly mediated by direct impact of pesticide applications. To overcome this concern, evidence from several sources were evaluated. For example, pesticide formulations used were of foliar application, applied during dry weather. Given the thick litter layer of grass and decaying vegetation on the soil surface, it is unlikely that significant amounts of pesticides were leached into the soil profile. We further examined the pesticide and their metabolite concentrations in soils immediately after sampling. No pesticide or metabolite residue was detected. Previous work on this site found no significant impact of insecticide applications on soil fauna (Allan and Crawley, 2011). Only populations of non-herbivore arthropods, ants and spiders were affected, which mainly feed to above-ground insects and other invertebrates. However, there is still a possibility of a long-term impact of pesticide application on some soil processes and microbial populations, which cannot be ruled out from the current experimental design. Previous reports on pesticide impact on soil microbial community structure and functions remain contradicting. For example, an earlier study (Ahtiainen *et*

al., 2003) reported no effect of pesticides on microbial biomass or respiration under field conditions. Contrastingly, another study (Eisenhauer *et al*., 2009) found that pesticides can affect both soil basal respiration and microbial biomass; however, authors argued that this impact of pesticide could be direct or indirect. They proposed a number of conceivable mechanisms of indirect impacts mainly through their effects on above-ground herbivory, and consequences for plant community structure, litter inputs and root exudations, which all can impact soil microbial communities. Our results provide support for the later mechanism, i.e. via reduced herbivory.

Fig. 4. Schematic representation of the effect of rabbit and invertebrate grazing on microbial ecological network interactions based on all functional genes as assessed by GeoChip analysis. Colours indicate different gene categories.

Synthesis and perspective

The results presented here provide strong evidence of the differential effect of vertebrate and invertebrate natural grazing on a number of key ecosystem characteristics. Previous studies using differences in vertebrate grazing intensity or simulated grazing have demonstrated that the response of soil communities and processes varies with intensity of herbivory (Guitian and Bardgett, 2000; Bardgett *et al*., 2001; Mikola *et al*., 2001).

Exclusion of invertebrate grazers had significant impacts on microbial biomass, community structure and genes associated with biogeochemical cycling. Several mechanisms have been proposed by which invertebrate grazing can influence below-ground communities directly and indirectly (Hunter, 2002). Invertebrate grazer can directly impact soil function and functional communities by returning significant amounts of N-rich faecal material to litter and soils (Grace, 1986), or by facilitating plants to double the rate of N release in soils (Schowalter *et al*., 1986). Diversity of invertebrates provides variable sources and complexity of nutrients (faecal or dead tissues), which are more easily decomposable and can stimulate decomposition of plant litter. Indirectly, invertebrates can change the quality and quantity of the litter inputs either via leaf abscission or via change in plant community structure (Chapman *et al*., 2003). They can also alter the amount and quality of root exudation, which is known to influence soil microbial communities (Bardgett *et al*., 1998). Additionally, invertebrate grazers can change the micro-climate (light availability, soil temperature and moisture) by impacting plant canopies (Hunter, 2002). Change in soil micro-climate can have significant impact on soil microbial community. Our data provide evidence that multiple or all-above mechanisms may operate simultaneously. Our experimental design rules out the impact of plant biomass on microbial communities and processes as standing vegetation was removed annually from the fenced plots. This suggests that invertebrate grazers-induced impacts on microbial community were either mediated directly as a food source or indirectly through changes in resource quality and/or functional changes in the composition of vegetation (Bardgett and Wardle, 2003). Indeed, higher levels of soil C and N in invertebrate-grazed soils support the above discussion. Significant changes in plant species diversity under invertebrate grazing have been reported previously across 1152 plots at this site (Allan and Crawley, 2011). A significant number of microbial genes associated with biogeochemical cycles were also responsive to invertebrate grazers, suggesting that above-ground invertebrates play a significant role in ecosystem function through impacts in energy flow and nutrient cycling pathways. While the role of soil-dwelling invertebrates in nutrient flows has been extensively studied (Edwards, 2000), the significance of above-ground invertebrates in nutrient flow pathways has received less attention. A recent study assessing the impact of above-ground invertebrate herbivores on soil CO_2 flux in a tall-grass ecosystem (Risch *et al*., 2013) reported that the exclusion of above-ground insect grazers led to reduced soil CO_2 emissions. Additionally, Babikova and colleagues (2013) recently

demonstrated the significant role of AM fungal networks in facilitating communication between plants under aphid attack, preparing plants for herbivore attack and thus potentially reducing herbivore damage. Together, these data suggest that above-ground invertebrates could have a pivotal influence on plant-soil feedback that are different from those imposed by larger vertebrate grazers. However, the above discussion should be interpreted with caution. Our experimental design does not allow to distinguish between direct effects of invertebrate grazing and long-term effect of applied pesticides. As discussed above, available evidence suggests minimal direct impact of pesticides; nonetheless, future works are needed to address this issue, and distinguish the direct impact of invertebrate grazing and long-term impact of pesticides in soil microbial communities and functions in order to quantitatively determine the contribution of above-ground grazing on below-ground functions and communities.

Rabbit grazing had significant impacts on bacterial, and to a lesser extent fungal, community structure obtained from TRFLP. This effect was mirrored in pyrosequencing data that showed dominant bacterial groups responding prominently to rabbit grazers, which could be associated with more rapid rate of nutrient turnover associated with animal excreta inputs in rabbit-grazed soils. By maintaining α-diversity, microbial communities were able to preserve the majority of tested physiological capabilities, and overall ecological functioning (heterotrophic respiration) remained less responsive. However, ecological network analysis provided further evidence that grazing is a key modulator of ecosystem functioning. Grazing-induced changes in soil functional communities and nutrient cycling could be more responsive to invertebrate grazers as suggested by significant increase in the abundance of genes associated with biogeochemical cycling, as well as connectivity in ecological networks.

Taken together, our data suggest that exclusion of rabbit and invertebrate grazing over the long term alters microbial community structure and functional potential that could have implications for energy and nutrient flows within systems. Furthermore, to address recent emphasis to link trait and functional group based approaches to predict the impact of diversity loss and community change on ecosystem functions, we used GeoChip analysis. This allowed us to directly evaluate impacts of the removal of vertebrate and invertebrate grazers on key functional groups associated with some key ecosystem functions and properties. We provide strong evidence of multi-trophic interactions (plant, vertebrate, invertebrate and microbial) in natural ecosystem and suggest that this interaction is a key modulator of overall ecosystem functioning. This finding encourages future studies to include more trophic levels (predators, birds, etc.) in experimental design to advance knowledge of multi-trophic interactions and consequences for ecosystem functions.

Experimental procedures

Nash's field

Nash's experimental field plots were established in 1992 to determine the impacts of rabbit and invertebrate grazing on grasslands ecosystems (Allan and Crawley, 2011). This long-established rabbit, insect and mollusc exclusion experiment is located on an acid mesotrophic grassland, MG5: *Cynosurus cristatus/Centaurea nigra* grassland, *Danthonia decumbens* subcommunity (Rodwell, 1992) in Silwood Park, near Ascot in South East UK. Full experimental details are previously described (Edwards and Crawley, 1999). The focus of this study was the impact of progressive grazer exclusion (molluscs, insects and rabbits) on below-ground communities and processes using a fully factorial (± insects × ± molluscs), slit plot design (± fencing to remove rabbit grazing) (Allan and Crawley, 2011). Full details on vertebrate and invertebrate exclusion are provided in the *Supporting information*. Un-limed subplots were used for this study, and four replicate cores were taken from each plot giving a total of 64 samples. Soils were sampled in June 2009, and the details are provided in the *Supporting information*.

Ecosystem functional measures

Soil- and substrate-induced respiration was measured using MicroResp™ (Macaulay Scientific Consulting, UK) to assess the effect of treatment on key ecosystem functions (Campbell *et al*., 2003). Briefly, soils were exposed to 15 different carbon sources (30 mg g^{-1} soil water) and the rate of respiration determined over a 6 h incubation period at 25°C as previously described (Campbell *et al*., 2003). Full details are provided in the *Supporting information*.

Microbial measures

Phospholipid fatty acids were used to measure microbial biomass. PLFAs were extracted from all samples using finely milled freeze-dried soils (500 mg) following the method of Frostegard and colleagues (1993). PLFAs were resolved by gas chromatography/mass spectrometry, and results are presented as microgram PLFA g^{-1} soil.

Microbial community structure was analysed by M-TRFLP and pyrosequencing. Total nucleic acids were extracted from all soils using MoBio PowerMax DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. DNA was used for TRFLP, GeoChip and Pyrosequencing analysis.

Multiplex terminal restriction fragment length polymorphism analysis was performed for all soils according to the method of Singh and colleagues (2006). Bacterial and fungal community profiles were analysed separately. *Pyrosequencing* was performed as previously described (Singh *et al*., 2014) on four replicate samples from each treatment. Fusion primers were used to amplify multiplexed, bar-coded 16S rRNA gene sequences. PCR products were purified, pooled and sequenced on a 454 GS FLX Titanium sequencer (Roche 454 Life Sciences, Branford, CT, USA). Following filtering for reads

with noisy bases (Huse *et al*., 2007), each sample was de-noised and processed using QIIME (Caporaso *et al*., 2010). De-noised data were aligned and classified through the Ribosomal Database Pyrosequencing Pipeline (http://pyro.cme.msu.edu/). Alpha diversity was assessed using Chao1 rarefaction curves, and community structure (β-diversity) was assessed on relative abundance data that had been log-transformed prior to multivariate analysis, as described below.

GeoChip 3.0 was used to determine the effect of treatment on functional gene diversity according to He and colleagues (2007; 2010) on + IM and − IM samples from fenced and grazed soils only (four replicates per treatment, $n = 16$). The GeoChip-based functional gene array covered 57 000 gene variants associated with carbon, nitrogen, phosphorus and sulfur cycles, as well as energy metabolism, antibiotic resistance and organic contaminant degradation. Simpsons and Shannon diversity indices were calculated for gene abundance data.

Statistical analysis

Two-way analysis ANOVA was used to determine the effect of rabbit grazers and invertebrate grazers, and their interaction, on chemical, functional and molecular measures. Linear regression was used to test the relationship between soil physical and chemical properties with biological measures. Further, multivariate analysis was used, whereby PC analysis, followed by canonical variate analysis of the first five PC scores, was employed to determine the influence of treatment on each set of analysis. All analyses were carried out using GENSTAT v16 (VSN International Limited, Hemel Hempstead, UK). GeoChip functional gene data were further analysed using an RMT-based conceptual framework to identify molecular ecological networks as previously described (Zhou *et al*., 2010b; Deng *et al*., 2012).

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